859

Purification of Human Red Cell Acetylcholinesterase

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Introduction

Purification of the acetylcholinesterase in human red blood cells approximately 300-fold has been reported briefly (1). An important step in the procedure is solubilization of the esterase in the red cell stroma by the use of the neutral surface-active agent polyoxyethylene sorbitan monolaurate (Tween 20).[2] Subsequent study led to modifications of the procedure that have increased the recovery of esterase to about 20%, and given a final product in dry form, free of Tween and of lipide.

Methods

Assay of Esterase

Acetylcholinesterase activity was determined manometrically at 38° with acetylcholine as the substrate. The assay volume was 3.5 cc. with a concentration of 0.04 *M* $MgCl_2$, 0.15 *M* NaCl, 0.025 *M* $NaHCO_3$, and 0.006 *M* acetylcholine. The system was equilibrated with 5% CO_2 in nitrogen. The concentration of substrate used on hydrolysis gave a linear release of CO_2 up to about 150 cu. mm. A unit of activity is defined as the amount of esterase that will release 1.5 cu. mm., equivalent to 0.067 μmole substrate, per 10 min. The specific activity is expressed as esterase units per milligram of protein.

Determination of Protein

The protein was determined with the biuret reagent and referred to the nitrogen content determined by the Kjeldahl method at various stages of the purification. Turbidity in the biuret reaction, due to Tween, was eliminated by shaking with a small amount of ether. After the esterase was obtained in soluble form, the total protein was determined from the ultraviolet absorption in a 1-cm. cell at 280

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[2] Mention of products in this paper does not imply endoresment or recommendation by the Department of Agriculture over similar products not mentioned.

mμ (optical density 1.8 is equivalent to 1.0 mg. protein/cc., referred to the nitrogen content). The hemoglobin present was determined from the absorption at 410 mμ (optical density 7.5 is equivalent to 1.0 mg. hemoglobin/cc.).

Electrophoresis

Electrophoresis on filter paper was performed by the method described by Kunkel and Tiselius (2). Two papers in layers were used; on one the protein distribution was determined by staining with bromophenol blue; on the other, the esterase was eluted with water from 2.5-cm. wide strips and determined by assay. Usually the recovery of esterase was about 80%.

Free electrophoresis was performed with the equipment developed by Tiselius.

Purification

Procedure. The starting material was packed human red blood cells obtained through the courtesy of Sharp and Dohme, Inc. Table I shows the steps in the method of purification, as well as the acetylcholinesterase activity per unit of protein and the recovery. The method is described in detail for the purification of 4 l. of the cells.

Four liters of the packed human cells is stirred into 24 l. water with a Premier mill for 2 min. while 272 ml. of 1.0 *M* acetic acid, which lowers the pH to 6.0, is added. The red cell wall is ruptured during this treatment. For smaller volumes, the stirring can be accomplished equally well with a Waring blendor. The vigorous stirring appears to be essential for the subsequent relatively rapid settling of the stromata (cell walls), which occurs at this pH. The stromata are permitted to settle for 8–24 hr. at a temperature of 7°. Subsequent washing operations are performed at this temperature. After the stromata settle, the supernatant fluid is decanted, and the stromata are washed by suspending in 40 l. of 0.01 *M* NaH_2PO_4. Settling of the stromata is rapid and will occur in less than 8 hr. The washing is repeated seven times in 1 week. The stromata are finally collected in a compact form by centrifuging and are brought to a volume of 4 l. with 0.1 *M* ethanolamine–HCl buffer, pH 9.6. The final pH is 8.5. The esterase activity lost on washing the stromata (Table I) is lost in the first decantation and may represent serum esterase in the serum adhering to the cells.

The stromata contain considerable hemoglobin in spite of the long period of washing. The nonlinear shape of protein adsorption isotherms and the increase in the elution of proteins with increase in alkalinity (3) suggested the following procedure for removing more hemoglobin. To each 4 l. of stromata, 400 ml. of 1.0 *M* cadmium acetate is added. The cadmium causes a flocculent precipitate of the stromata which can readily be sedimented in a centrifuge. The sediment is washed once by resuspending it in the equivalent volume of water and is finally restored to a volume of 3.8 l. with ethanolamine buffer.

The cadmium-precipitated stromata are immediately solubilized by the following procedure: Half the solution is convenient to handle at one time: 1.9 l. of the esterase is mixed with 100 ml. of 2.3 *M* K_2HPO_4, 100 ml. of Tween 20, 540 ml. of 4.6 *M* ammonium sulfate, and 35 ml. toluene. The mixture is thoroughly stirred and then centrifuged. A top solid, fatty layer is obtained, composed of Tween,

toluene, and protein. From 75 to 80% of the esterase is in this layer and 20–25% in the subnatant fluid. The fatty layer is removed, adjusted to 700 ml. with ethanolamine buffer, and dialyzed for 3–4 days at 7° to break the emulsion. the esterase can be stored in this form.

After the 4-day dialysis, the esterase preparation is turbid and contains large amounts of Tween. Much of the Tween is removed, and some purification of the

TABLE I

Purification of the Acetylcholinesterase in Human Red Blood Cells

Fraction	Description	Activity units/mg. protein	Recovery of initial activity %	Purification
1	Human red blood cells	4.5	100	—
2	Stromata in 0.1 *M* ethanolamine buffer, pH 9.6, after hemolysis of cells with dil. acetic acid, and washing of stromata in 0.01 *M* NaH_2PO_4	100	85	22
3	Cadmium acetate ppt., suspended in ethanolamine buffer		60	
4	Lipide layer after treatment of stromata ppt. with Tween 20, toluene, and ammonium sulfate	60	total 75–80	
4*a*	Subnatant fluid from 4, discarded	15–20		
5	Esterase, after 4-day dialysis and pptn. of bulk of Tween with ammonium sulfate	400	40	89
6	Esterase after calcium phosphate treatment and clarification with Filter-Cel		25	
7	Purified esterase, dry powder, after being dried in frozen state and extracted with acetone, ethanol, and ethyl ether	1100	20	245

esterase is obtained by adding ammonium sulfate to a concentration of 1.06 *M*. The esterase is recovered and concentrated by precipitating at 3.2 *M* ammonium sulfate and subsequently dialyzing free of salt.

The esterase is purified further by treating with $Ca_3(PO_4)_2$, which removes protein with little loss of esterase. Uniform $Ca_3(PO_4)_2$ preparations have been difficult to prepare, but the most suitable product has been obtained by suspending the calcium phosphate in 0.5 *M* potassium phosphate for 18 hr. at pH 7.0 and sedimenting and washing with water just before use. This procedure is applicable to either wet or dried preparations of calcium phosphate. The amount of calcium phosphate required for maximum purification with minimum loss of esterase (ap-

proximately 0.4 g./10 ml. esterase) is determined by trial with small portions of each preparation.

After removal of the calcium phosphate, the esterase is still slightly turbid. Clear solutions can be obtained by adding 2% Filter-Cel and filtering. The clear solutions are concentrated by precipitation at 3.20 *M* ammonium sulfate, and dialyzed against dilute ethanolamine (0.3 ml./10 l. water) which maintains an alkaline pH reaction. If the solution contains much hemoglobin (20% or greater), the calcium phosphate treatment is repeated at a protein concentration of 0.1–0.2%. This procedure usually reduces the hemoglobin content to approximately 10%.

The esterase preparation is concentrated by ammonium sulfate precipitation, followed by dialysis and dried in the frozen state. The dried esterase from 4 liters of red cells is triturated with approximately 200 cc. of acetone and centrifuged, and the acetone is decanted. This treatment is repeated with ethanol (absolute) and ethyl ether, and the solid is finally dried in a stream of air. The extractions are carried out at about 5°; loss of esterase is about 10%.

Results

The dry powder resulting from the procedure described contained 13.0% nitrogen, which was increased to 14.1% by correction for 2.0% ash and 6.0% volatile matter. The specific esterase activity was 1000–1200 units/mg. protein. The over-all purification was about 250-fold. In some instances, the purification was as high as 400-fold.

The Tween was removed from preparations dried in the frozen state by extraction with acetone alone. After this treatment, however, the dry solid contained only 7–8% nitrogen. Further study showed that these preparations contained 30–35% of lipide that could not be extracted with acetone, petroleum ether, or ethyl ether, but it could be extracted with *n*-butanol or ethanol. The extraction of this lipide, presumably red cell lipide, with an alcohol-type solvent appears to be due to a specific solvent effect. It should be emphasized that solutions of the esterase were clear even when this lipide was still present.

In electrophoresis on filter paper, the esterase moved toward the positive electrode at pH 6–9, and the movement changed little with change in pH in this range. The hemoglobin color in these preparations was puzzling for it moved toward the positive electrode even at pH 6.0. As expected, pure hemoglobin on electrophoresis at pH 6 moved toward the negative electrode. On electrophoresis at pH 8.5–9.5, the esterase activity and the bulk of the protein were sharply separated, particularly with lipide-free preparations. For example, on Whatman No. 3MM paper, in 18 hr. at 6 v/cm. in 0.1 *M* glycine buffer, pH 8.8 at 20–25°, the esterase was localized at 8–12 cm., whereas the bulk of the protein

was distributed from 14 to 24 cm. The esterase eluted from the paper was threefold purer than the starting material. (Substances on the paper that absorb ultraviolet light must be considered when estimating protein by ultaviolet absorption.)

Discussion

The procedure just described gives a dry preparation of a soluble acetylcholinesterase in good yield (10–20 %). The preparations are regularly about twice as active as cobra venom, hitherto the only preparation of acetylcholinesterase available in dry form (4).

Probably 1 % or less of the red cell preparation is cholinesterase protein. Cholinesterase of greater purity has been prepared from the esterase-rich, but less available, electric eel. The literature describing the purification of acetycholinesterase has been summarized in a recent review (5).

The ability of Tween 20, a neutral surface-active agent (other reagents of this type have not been tested), to solubilize the esterase is not surprising. Lysolecithin has been used to solubilize erythrocyte acetylcholinesterase (6), and recently it was found that neutral surface-active agents solublize heart and brain cholinesterases (7). Ionic surface-active agents inactivate cholinestereases (7). Recently, Tween 21 has been used in the purification of pig brain acetylcholinesterase (8); however, in this case the concentration used was only 0.14 %. The nature of the Tween effect is not known, although presumably a dissociation of a lipide–protein complex occurs. It is of interest that Tween 20 will hemolyze red blood cells (9).

The use of cadmium acetate and retention of the esterase in the Tween layer at 20–25° differ from the previously described method (1). Previously, the Tween mixture was filtered, and about 50 % of the esterase was found in the filtrate. It had been found that filtration at 7° gave slightly more esterase in the filtrate than filtration at 20–25°. When cadmium acetate was used, temperature caused an extreme difference in the amount of esterase in the filtrate; at 20–25° only about 10 % of the esterase was in the filtrate, whereas at 7° this increased to 50 %. It was concluded that the cadmium salt had altered the properties of the complex mixture, probably by binding a lipide, which made it possible to retain the esterase in the Tween-containing layer at 20–25°. All the cadmium appeared to be separated from the esterase mixture as a dense sediment, probably a lipide salt which is sedimented when the

mixture is centrifuged. When this sediment was resuspended, it had a striking silky appearance, typical of fatty acid–metal precipitates. The esterase was partly inactive in the cadmium precipitate, but on subsequent dissociation the activity was restored (Table I).

Organic solvents have been found to be extremely destructive to acetylcholinesterase in aqueous solutions. The resistance of the esterase in the dried state to organic solvents was in striking contrast. A similar observation has been made of the acetylcholinesterase in brain tissue (10). Recently, a purification of the cholinesterase in ox red cells was described briefly (11) in which the stability of the dried esterase was recognized. A key step in the method was freeze-drying of the stromata followed by extraction with butanol. After additional fractionations with ammonium sulfate and Lloyd's reagent, purification of 250–400-fold was attained.

The isoelectric point of horse red cell cholinesterase has been reported to be 4.7 (12). The esterase in this instance, however, was still bound to the stromata, and the isoelectric point reflects the composition of the stromata rather than the esterase. The present studies indicate that the isoelectric point of the free esterase lies below pH 6.0. Studies at lower pH values are hampered by the instability of the enzyme in this region.

Summary

The acetylcholinesterase in human red blood cells was extracted from the stromata by means of the surface-active substance polyoxyethylene sorbitan monolaurate (Tween 20). After a series of purification steps, preparations dried in the frozen state were freed of Tween by extraction with acetone and freed of lipide by extraction with *n*-butanol or ethanol. The dry powders, representing a 250-fold purification, were stable at 7°. Electrophoresis on paper showed that the isoelectric point of the esterase lies below pH 6.0.

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